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14. ABSTRACT

Many challenges exist in the current management of metastatic breast cancer as there are fewer recognized therapeutic strategies. Therefore, a better understanding of the molecular events in the metastatic process is critical. Several reports have described correlation of hyaluronan (HA) with initiation and progression of various types of epithelial cancers. The HA synthase (HAS) isoforms encode the enzymes that produce and deposit HA while the hyaluronidase (HYAL) genes code for enzymes that degrade HA and their expression is dysregulated in various tumors as a result of transcriptional and epigenetic changes that accompany progression. In this report, demonstrate that Has2/Has3 knock-down and LYVE1 overexpression modulated mammary tumor growth and spontaneous metastasis. Moreover, expression of CCR7 had no effect on primary tumor growth, but affects lymph node metastasis and CCL21-induced chemotaxis. Hyaluronan did not affect CCL21 expression in mammary tumor cells. Lymph endothelial cells constitutively express CCL21, which is not affected following treatment with hyaluronan. Together, these data suggest an important role of HAS2, LYVE1 and CCR7 in a complex interaction between tumor cells and lymph endothelial cells during mammary tumor growth, angiogenesis, invasion, and lymph node and distant metastasis.

15. SUBJECT TERMS

Hyaluronan Synthase, Tumor Growth, Metastasis, Breast Cancer

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Progress Report

Lymph node metastasis represents one of the first steps in breast cancer metastasis. At the time of diagnosis, majority of breast cancer patients have developed lymph node (LN) metastases, which is an important prognostic indicator. The mechanism(s) regulating lymphatic invasion and metastasis in breast cancer are currently unknown. A better understanding of the biology of malignant cells and lymphatics in LN metastasis has important therapeutic implications in breast cancer.

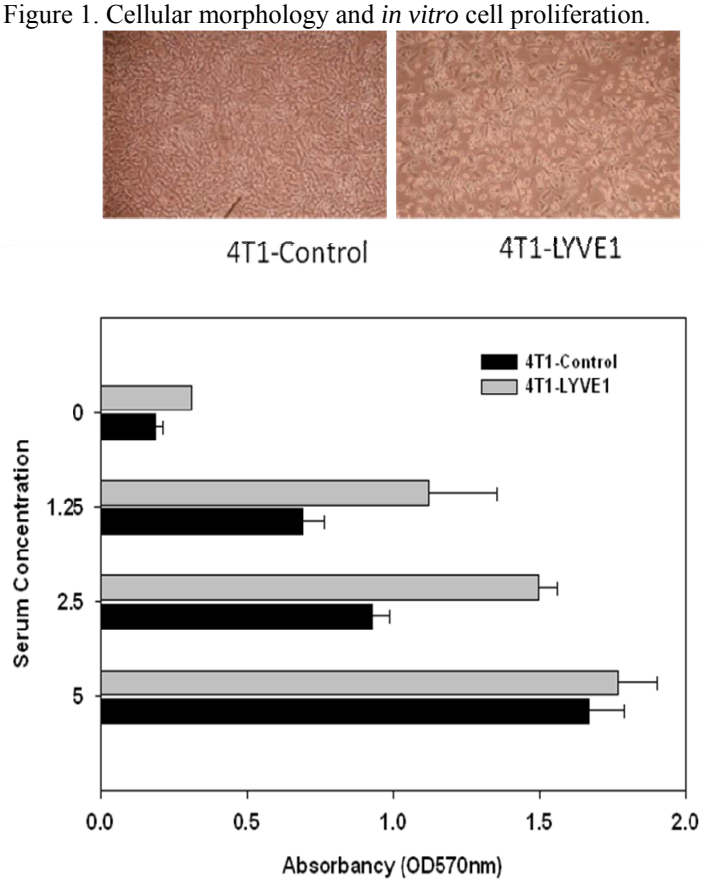
Objective/Hypothesis: The lymphatics, an extensive network of vessels, play a key role in immune surveillance, transport and recirculation of extracellular matrix (ECM) components such as hyaluronan (HA) from the interstitial fluid. The transport of HA through lymphatics is exciting, as this glycosaminoglycan has already been implicated as substrate for both leukocyte migration and tumor dissemination. Malignant cells produce and shed HA into ECM and a higher level of HA in the tumor interstitium predicts poor survival of breast cancer patients, possibly because of enhanced invasion/metastasis. Recent studies have identified a specific HA receptor, LYVE-1, primarily on lymph endothelial cells (LEC). Sequestration of HA through interaction with LYVE-1 facilitates HA transport and degradation within the lymphatics. Exploitation of this physiological pathway may provide a conduit for malignant tumor cells to metastasize to LN. Recent studies suggest that HA-LYVE-1 interaction could allow up-regulation of secondary lymphoid tissue-chemokine (SLC/CCL21), a chemokine that induces migration of inflammatory leukocytes to lymph nodes. CCL21 is primarily expressed in LECs and functions as a chemoattractant for CCR7-expressing dendritic cells and T cells. We hypothesize that exploitation of HA-LYVE-1 interactions by breast cancer cells allows their preferential LN metastasis mediated by CCL21. In this current proposal, we will test this unique concept which will provide an insight into interactions of breast cancer cells with LECs, their translocation through LECs and establishment as LN metastasis. In this Concept Award application, we will test the hypothesis that HA-LYVE-1 interaction resulting in LEC production of CCL21 regulates LN metastasis in breast cancer. Two specific aims are proposed.

Specific Aims:

1) Test the hypothesis that expression of HA and LYVE-1 on breast cancer cells and LECs and their interaction regulate LN metastasis.

2) Test the hypothesis that binding of HA to LYVE-1 regulates SLC/CCL21 production in LECs, which functions as chemoattractant for CCR7-expressing breast cancer cells.

Figure 1. Cellular morphology and *in vitro* cell proliferation.



Results.

Expression of HA and LYVE-1 on breast cancer cells and LECs and their interaction regulate LN metastasis.

1. We analyzed the basal expression of LYVE1 and HA and Has1, Has2, and Has3. 4T1 cells express high levels of Has1 and Has2 and HA. We did not observe LYVE1 expression in 4T1 cells.

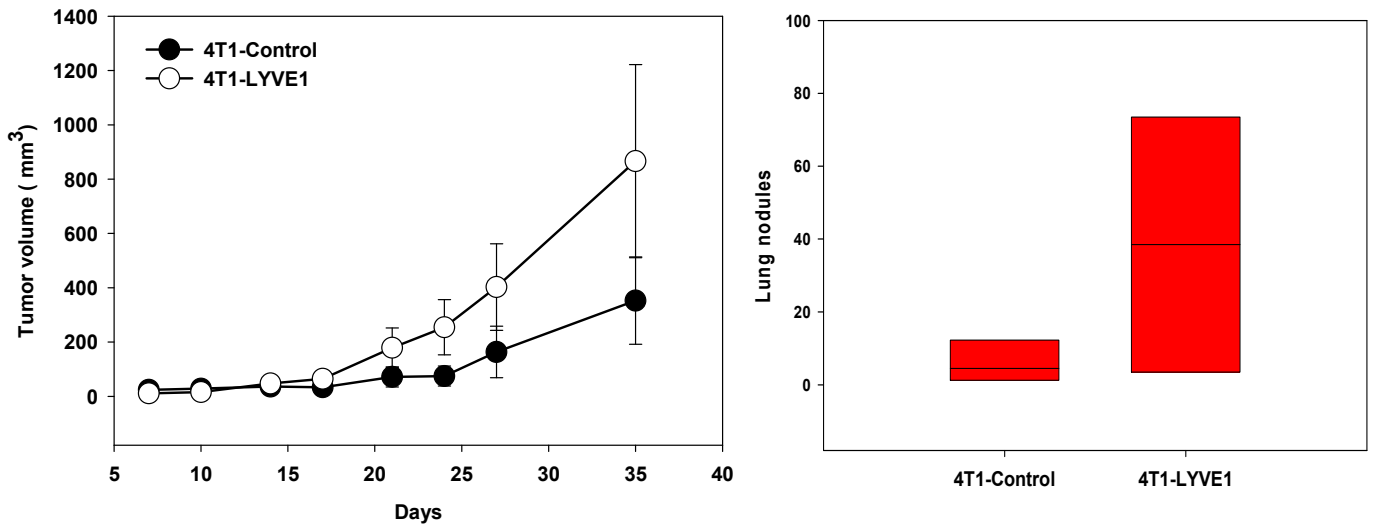
2. We stably transfected 4T1 cells with mammalian expression vector containing LYVE1 cDNA to generate stable LYVE1 expressing 4T1 cells. We analyzed expression of LYVE and *in vitro* phenotypes of these cells. LYVE1 expressing cells showed growth advantage at lower serum concentrations as compared to control vector transfected cells. We observed a morphological distinction in

4T1-LYVE1 cells (**Figure 1**).

3. We transfected 4T1 cells with shRNA vector targeting Has2 and Has3 expression. Following selection to derive stable clones, cells were examined for Has2, Has3 and HA expression using realtime RT-PCR.
4. 4T1-LYVE1 and 4T1 control vector cells were injected into mammary fat pad of BALB/c mice and tumor growth and metastasis was monitored.

We observed enhanced tumor growth and spontaneous lung metastasis in animals injected with 4T1-LYVE1 cells as compared to 4T1-control cells (**Figure 2**). These data suggest that ectopic expression of LYVE1

Figure 2. Enhanced Tumor Growth and Spontaneous Lung Metastasis in LYVE1 Expression Mammary Tumor Cells.

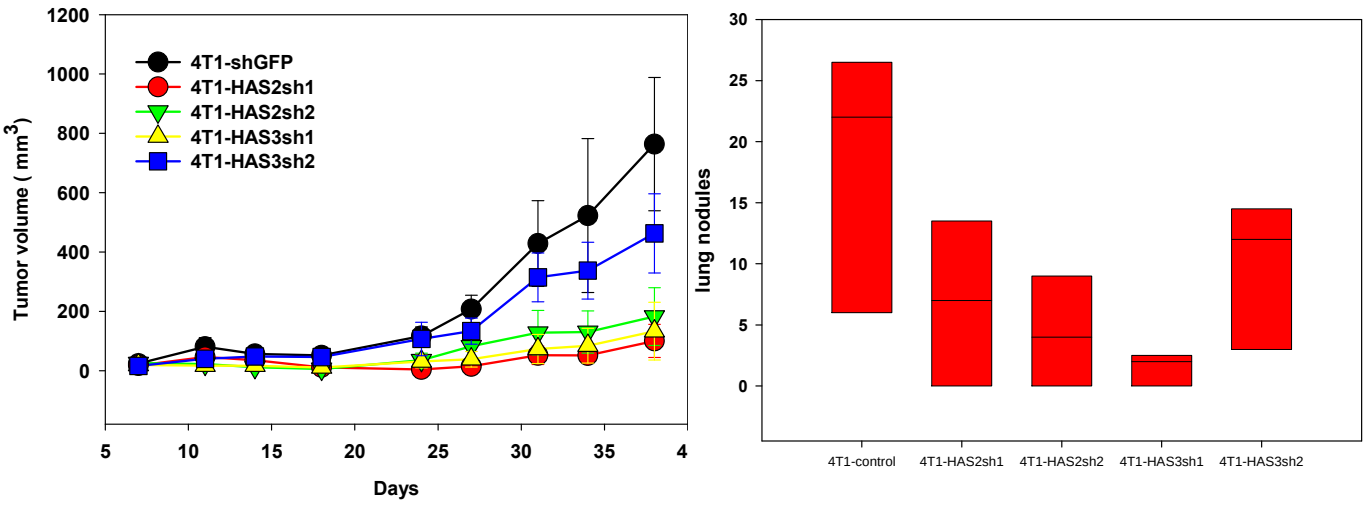


enhanced tumor growth and metastatic potential in mammary tumor cells.

Has2 and Has3 expression in mammary tumor cells modulates tumor growth angiogenesis and metastasis

Several reports have described correlation of hyaluronan (HA) with initiation and progression of various types of epithelial cancers. The HA synthase (HAS) isoforms encode the enzymes that produce and deposit HA while the hyaluronidase (HYAL) genes code for enzymes that degrade HA and their expression is

Figure 3. Inhibition of Tumor Growth and Spontaneous Lung Metastasis in Has2 and Has3 knockdown Mammary Tumor Cells.

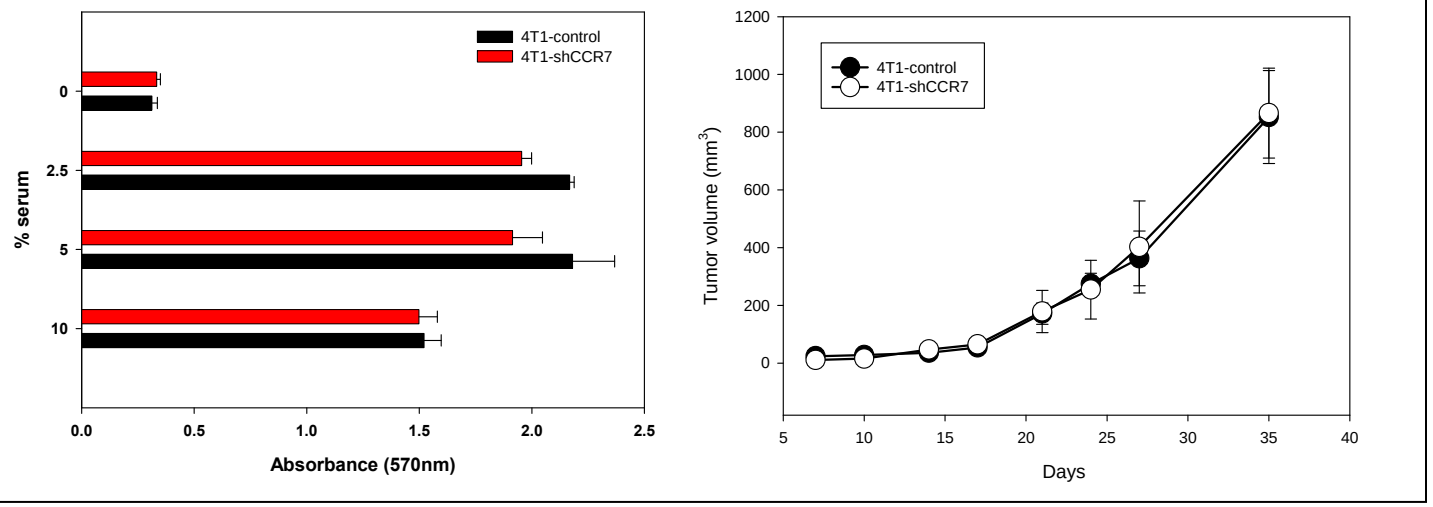


dysregulated in various tumors as a result of transcriptional and epigenetic changes that accompany progression. In this report, we examined the expression of HAS1, HAS2 and HAS3, HYAL1 and HYAL2 in mammary tumor cell with different metastatic potential (4T1, highly metastatic; Cl66, moderately metastatic; Cl66M2, low metastatic). We observed increased expression of HAS1, HAS2 and HAS3 as well as HAYL 1 and HAYL2 in aggressive mammary tumor cells. Next we stably knocked-down HAS2 and HAS3 expression in 4T1 cells using small hairpin mRNA (sh-RNA) vectors and analyzed cell proliferation, migration, tumor growth and metastasis. We observed inhibition of *in vitro* cell proliferation and migration in 4T1 cells knocked-down for HAS2 (4T1-HAS2sh) and HAS3 (4T1-HAS3sh) as compared to cells transfected with vector control (4T1-controlsh). Furthermore, we observed inhibition of *in vivo* tumor growth and spontaneous lymph node and lung metastases in animals implanted with 4T1-HAS2sh cells as compared to 4T1-controlsh (**Figure 3**). In addition, we observed inhibition of tumor cell proliferation and neovascularization, and increased apoptosis in 4T1-shHAS2 tumors as compared to 4T1-controlsh tumors. Together, these data demonstrate an important role of HAS2 in mammary tumor growth, angiogenesis, invasion, and metastasis.

CCR7 expression in mammary tumor cell and tumor growth and lymph node metastasis

We generated three stable isogenic 4T1 cells, 4T1-control, 4T1-shCCR7, expressing different levels of CCR7 by stable transfection of shRNA targeting CCR7. We used these cells to examine whether CCL21 modulates chemotaxis and migration of CCR7 expressing mammary tumor cells. We examined in vitro and in vivo growth of 4T1-control and 4T1-shCCR7 cells. We did not observe any significant difference in *in vitro* cell proliferation and *in vivo* tumor growth (**Figure 4**).

Figure 4. Tumor Growth *in vitro* and *in vivo* following CCR7 knockdown in Mammary Tumor Cells.



Interestingly, we observed lower incidence of lymph node metastases in mice injected with 4T1-shCCR cells as compared to 4T1-control. These data suggest that CCR7 modulation had no effect on primary tumor growth, but affects lymph node metastasis.

Effect of CCL21 on CCR7 expressing mammary tumor cell chemotaxis

In the nest set of experiments, we examined whether CCR7 modulation affect the migration of mammary tumor cell in response to CCL21. We observed a significant increase in chemotaxis of CCR7 expressing 4T1-control cell in response to CCL21. The levels of CCL21-induced chemotaxis in 4T1-sh1CCR7 and 4T1-sh2CCR7 cells were significantly lower as compared to 4T1-control cells suggesting that binding of CCL21 to CCR7 in mammary tumor cells regulate chemotaxis.

Effect of hyaluronan on mammary tumor cell CCL21 production

We examined whether modulation of hyaluronan expression affect CCL21 expression in mammary tumor cells. We used our mammary tumor variants expressing different levels of Has2 and 3 genes and examined CCL21 using ELISA. We did not observed any significant difference in secreated or cell-associated CCL21 in mammary tumor cells with different levels of hyaluronan.

Effect of hyaluronan on lymph endothelial cell CCL21 production

Next we examined direct effect of hyaluronan on LECs expression of CCL21 by ELISA. LECs constitutively express CCL21. Treatment of LECs with different concentrations of hyaluronan did not alter secretion of CCL21 in LECs. These data suggest that constitutive expression of CCL21 by LECs might be sufficient for chemotaxis of CCR7 expressing mammary tumor cells.

Together these data suggest a complex interaction between LECs and mammary tumor cells in lymph node metastasis.

Relevance: These studies will provide unique insights into a causal relationship between HA-LYVE-1 and SLC-mediated regulation of LN metastasis in breast cancer. The knowledge gained from these studies will provide a foundation to develop diagnostic markers and novel therapeutics to inhibit early LN metastasis in breast cancer.

Publication: Abstract:

Varney ML, Nannuru KC, and **Singh RK**. Hyaluronan Synthase Expression in Mammary Tumor Cells Modulates Tumor Growth Angiogenesis and Metastasis. Annual Meeting of American Association for Cancer Research 2010.